REMARKS

Examiner Bland is thanked for her careful review and examination of the present patent application. In response thereto, applicants have entered certain amendments to the claims and request reconsideration of the claim rejections.

The independent claims have been amended to recite that a majority of the bonds in the reaction products are 1,4-bonds and that the product is substantially digestible via mammalian enzymes. Full support for these amendments can be found, *inter alia* at page 11 of the specification. It is noted that the independent claims of the application specify that the starting material in the extruder is a mixture of *malto-oligosaccharides.* In a malto-oligosaccharide, essentially all of the carbohydrate linking bonds are alpha 1,4-linking bonds. These bonds are essentially completely digestible and completely subject to enzymatic hydrolysis via mammalian enzymes. In the extruder, the malto-oligosaccharide is derivatized to result in a carbohydrate product with bonds other than alpha 1,4- linking bonds, these including, for instance 1,2-linking bonds.

At page 11 and elsewhere, the specification indicates that the reaction product may have a range of properties, which properties depend on the conditions in the extruder and mix of starting materials. In some embodiments, "the product may be substantially inert to digestion by the mammalian enzymes." In other embodiments, however, the specification teaches that the product "alternatively may be substantially digestible, but digestible slowly relative to glucose." This is a result of relatively low levels of chemical modification of the starting material. In such a product, although there are some non-1-4 linking bonds (e.g., 1-2, 1-3 bonds), a majority of bonds will be 1,4-bonds. It is this product that is claimed herein.

The various claim rejections will be addressed in turn.

Section 112

The Examiner's Section 112 rejections are now moot. The numerical digestibility limitations have been excised from the claims.

At the interview, the Examiner raised the question as to the use of the term "substantially." There is nothing indefinite about this term as used in the pending claims.

As set forth in *Andrew Corporation v. Gabriel Electronics, Inc.,* 847 F.2d 819 (Fed. Cir. 1988), where the claim at issue used the phrase "substantially," a patentee is permitted to define her invention using terms of relativity:

> Patentability is not measured against the closest point on the road to invention. Much technological change that meets the criterion of unobviousness, when viewed in light of the prior art, has a fuzzy boundary at its point of origin. Technological differences from prior art usually become more pronounced with distance from the boundary, but the changes may become manifest gradually. Indeed, the location of the boundary may well change with the available precision of measurement.

See M.P.E.P. 2173.05(b): "The fact that claim language, including terms of degree, may not be precise, does not automatically render the claim indefinite under 35 U.S.C. 112, second paragraph. . . . Acceptability of the claim language depends on whether one of ordinary skill in the art would understand what is claimed, in light of the specification."

In this case, the specification discusses a number of products that are essentially indigestible (e.g. FIBERSOL, one of the very products that forms the basis of the Section 103 rejection). The specification further indicates that an object of the invention, at least in some embodiments is to produce a product that is substantially digestible. These teachings serve to demarcate the claimed invention from the essentially indigestible products of the prior art with sufficient specificity under 35 U.S.C. 112. Accordingly, no new Section 112 rejection should be issued.

Section 102 rejection over Meyers, et al.

It is respectfully submitted that the Examiner continues to misconstrue the Meyers reference. This reference purports to disclose a use of FIBERSOL in chewing gum. This product is discussed and differentiated in the specification of the present application, and

was evaluated by Dr. Mungara in the previously submitted Declaration, and, as set forth below, is not the same as the claimed product.

FIBERSOL is sold by Matsutani America, and is described in numerous Matsutani patents. Meyers discloses simply a use of FIBERSOL. The Meyers patent is silent as to the method of preparation of FIBERSOL, and is largely silent as to its properties (although Meyers does recognize this product as being an "indigestible dextrin," column 3, lines 15-18).

Attached herein as Exhibits A and B hereto are printouts from Matsutani America's website. These exhibits provide more detail as to FIBERSOL. In Exhibit A, FIBERSOL is characterized as a soluble dietary fiber (90% minimum fiber on a dry solids basis). The designation as "fiber" connotes an indigestible product, and a minimum of 90% fiber signifies that essentially the entirety of the FIBERSOL product is not subject to digestion. Exhibit A references this expressly: "The human digestive systems effectively digest only alpha 1,4 linkages; therefore the other linkages render the molecules resistant to digestion." In Exhibit B, Matsutani further confirms that, per 100 grams of FIBERSOL, there are 95 grams total carbohydrates and 90 grams of dietary fiber. This exhibit confirms that essentially the entire product is indigestible. FIBERSOL unquestionably is different from the product claimed in the present application.

The indigestibility of FIBERSOL is confirmed by Dr. Mungara. In his Declaration, he reported digestibility data for FIBERSOL and for two embodiments of the present invention. In this demonstration, he established that the 4-hour digestibility data for FIBERSOL was about an order of magnitude less than the two products evaluated.

FIBERSOL is itself discussed in the specification and is differentiated from the present claims by the discussion of substantial digestibility.

Thus, Meyers cannot anticipate the present invention.

Section 102 Rejection over Yoshida

Applicants continue to traverse the rejection over Yoshida. In Yoshida, ***no bonds are created***. There will be nothing other than alpha 1,4- and alpha 1,6- bonds. This is in contradistinction to a reaction performed in an extruder in which other types of bonds including 1,2-, 1,3-, and both alpha and beta bonds will be created.

The Examiner asserts that "regarding claim 35, the limitation of how the extrusion is performed does not have any patentable weight to the composition claimed in claim 1." This is simply incorrect. Again, the reaction in an extruder of necessity will create a variety of bonds other than alpha 1,4- and alpha 1,6- bonds, and hence of necessity will create a product that is manifestly different from the product disclosed by Yoshida. Put another way, the fact that an extruder is involved indeed is of "patentable weight" because the extruder creates a product that is necessarily different from the product of Yoshida. The Yoshida reference is simply inapposite and the Examiner is respectfully requested to withdraw the rejection over same.

Section 102 Rejection over Fouache et al.

The product of Fouache is different from the claimed product. This is evident both from the data provided by Fouache and from Dr. Mungara's declaration.

At column 2, column lines 45-61, Fouache discusses the "calorific value" of the disclosed product. Fouache describes his product as having a calorific value lower than 2.5 kcal/g. Of this, most of the calorific value consists of an "indigestible fraction" that is fermented bacterially in the large intestine (and hence that is not subject to digestion via mammalian enzymes). This "indigestible fraction" is indicated as providing 2 kcal/g of the 2.5 kcal/g digestibility. Fouache thus discloses a product that has an enzymatic digestibility of 0.5 kcal/g. This translates to about 12.5% digestibility (based on 4 kcal/g carbohydrate). This general teaching is confirmed by Dr. Mungara. He evaluated a commercial NUTRIOSE product, and found it to have a digestibility of 15%.

The Fouache reference indicates that the product is characterized by "a content of glucosidic linkages 1→4 can be between 42 and 50%." This teaches that the Fouache product does not contain a majority of 1,4- linkages. The product of Fouache is hence different from the claimed product, and Fouache does not anticipate any pending claim.

The Examiner has questioned certain data in Fouache. As an initial matter, the data presented in Fouache is presented differently from the data presented in the Mungara declaration, and the data cannot be directly compared. Specifically, Dr. Mungara differentiated between the various types of carbohydrates in the molecules, while the Fouache reference does not. For instance, Dr. Mungara counts a 1,4-linkage separately from a 1,2,4-linked carbohydrate. Fouache counted these linkages as a single 1,2 and a single 1,4 linkage, and did not count this 1-4 linkage separately from other 1,4-linkages when reporting the total number of 1,4-linkages. For purposes of digestibility, however, the differences between a simple 1,4- linkage and a carbohydrate that has multiple linkages are substantial. Recalculating the Mungara data and presenting it in the form provided by Fouache yields the following:

	NUTRIOSE	FIBERSOL	Sample 1	Sample 3
1,2- bonds	9.8	9.9	7.8	5.9
1,3- bonds	9.9	8.3	7.8	6.4
1,4- bonds	49.6	51.5	59.7	67.2
1,6- bonds	30.7	30.1	24.3	19.5

More fundamentally, however, the susceptibility of a carbohydrate to enzymatic hydrolysis is not defined solely by the number of 1,4- bonds. This is apparent from a review of the FIBERSOL data (roughly the same number of 1,4 bonds when reported in the above format as NUTRIOSE, but very different digestibility profiles). The digestibility of a carbohydrate is a function not only of the type of bonds but also of the structure of the carbohydrate and the position of the bonds relative to other bonds in the carbohydrate. Some portions of a carbohydrate may be sterically unavailable to enzymes. For instance, a carbohydrate product that included bonds that are entirely linked via 1-4 linkages with no other types of linkages, is likely to be essentially 100% digestible via mammalian enzymes. When indigestible bonds, such as 1,3 bonds, are introduced into the carbohydrate structure, a mammalian enzyme may be unable to digest the carbohydrate. This is true even if the majority of the bonds in the carbohydrate product are 1,4 bonds.

In any case, the product of Fouache et al. does not meet the claim limitations, and does not anticipate any of the pending claims.

Section 102 Rejection over Stahl

The newly sited Stahl reference is not relevant to the claims of the present application. Stahl discloses several carbohydrates that have been modified with various enzymes. In Example A1, which is the portion of Stahl's specifically relied upon by the Examiner, maltodextrin was derivatized with glucose and a *leuconostoc* enzyme. *Leuconostoc* enzymes are well known in the art. As with most other enzymes, *leuconostoc* enzymes are very specific in their action. A product resulting from a leuconostoc-catalyzed reaction will consist almost exclusively of a carbohydrate that is linked with alpha-1,6- bonds. In some embodiments there may be small portions of alpha-1,2- or alpha-1,3- bonds. Nonetheless, the enzymatic reaction is very specific and characteristic, and results in a product that is very well defined.

Attached as Exhibit C is a copy of an article by Quirasco et al., "Induction and Transcription Studies of the Dextransucrase Gene in *Leuconostoc mesenteroides* NRRL B-512F," *Applied and Environmental Microbiology*, , Vol. 65, No. 12, December 1999, p. 5504-5509. As indicated therein, "*[l]euconostoc mesenteroides* NRRL B-512F produces an extracellular DS that synthesizes a soluble polymer, 95% of which is composed of alpha -(1-6) linkages in the main chain and 5% of which is composed of alpha -(1-3) branched linkages." Other *leuconostoc* enzymes would produce other variants, but in each case the enzymatic reaction would produce a specific carbohydrate with a well-defined carbohydrate structure.

This specific product is different from the product of the claimed invention. As prepared in an extruder, the product will be different from the product of Stahl in several respects. First, the product in the extruder will include some bonds that are not alpha bonds, but instead are beta bonds. These would simply not be produced with the *leuconostoc* enzyme. Second, whereas with a *leuconostoc*-catalyzed reaction, essentially all of the bonds in the resulting product will be 1,6 bonds, the bonds prepared in the extruder will be more randomly distributed.

The Examiner has pointed to claim 4 of Stahl in the translation. Although it is not clear from this rough translation, Stahl appears to disclose a substantial number of

enzymes and a wide variety of reaction products. Claim 4 appears to indicate that the variety of enzymatic reaction products can have a variety of bonds, these including, in various embodiments, 1,2-, 1,3-, or 1,4- bonds. In other words, claim 4 of Stahl appears to address the notion that various enzymes can produce various types of reaction products. Nonetheless, nothing in Stahl can constitute a disclosure of the present invention. Again, enzymatic reactions tend to be very *specific*. Each enzymatic reaction will produce a carbohydrate of a very specific profile. This is dissimilar to the extrusion reaction claimed here, where the bonds formed in the extruder are more randomly produced.

The undersigned acknowledges the doctrine expressed by the Examiner that "the patentability of a product does not depend on its method of production." Nonetheless, where the claimed product is very substantially different from the prior art – in this case, because the claimed product has different types of bonds and a different distribution of linkages – the cited art cannot be the basis of the claimed rejections.

Section 103 Rejections

The Section 103 rejections should be withdrawn. Unlike the anticipation analysis under 35 U.S.C. 102, for a Section 103 rejection, the general teachings of the references must be taken into consideration. In this case, the references not only fail to anticipate the claimed invention, they teach away from the claimed invention, and the Section 103 rejections must be withdrawn,

With regard to the Meyers reference, this teaches the provision of a product that is substantially less digestible than the product claimed in the present application. This constitutes a strong teaching away from the claimed invention. Fouache is likewise not useful in a Section 103 rejection because Fouache teaches a different carbohydrate prepared in a different way to result in a different number of digestible linkages. It is unclear to the extent to which Yoshida is deemed to be relevant for a Section 103 rejection, but again Yoshida would teach away from the present invention in that it discloses a product that includes only alpha 1,4 and 1,6 bonds. Finally, Stahl also teaches away from the present invention because it provides for a number of specific

enzymatically catalyzed reaction products. Each such reaction product is highly specific, and none of the Stahl products are a mixture of more random bonds as would be produced in an extruder. The Stahl reference (as does any number of other references) provides for enzymatic modification of a carbohydrate. A person skilled in the art would be led away from the use of something other than an enzyme by Stahl, because the employment of an enzyme is used in circumstances where a specific reaction product is desired.

In short, none of the references individually are useful in connection with a Section 103 rejection. When the references are considered together, the case for a Section 103 rejection becomes even worse. Stahl is incompatible with any of the other references. Yoshida does not teach the formation of bonds and likewise does not appear to be compatible with any of the other references. Meyers teaches a substantially indigestible product. The references cannot be fairly considered together, and even overlooking this, the combination of references does not lead to the claimed invention.

Double Patenting

Applicants traverse to the extent that the identified applications have not been patented.

Conclusion

For these reasons, withdrawal of all the rejections is respectfully requested.

Respectfully submitted,

Dated: 11/13/03

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EXHIBIT A

Matsutani America, Inc.

What is Fibersol®-2?

Fibersol®-2 is a soluble dietary fiber (90% min. dsb). Fibersol®-2 is produced from corn starch by pyrolysis and subsequent enzymatic treatment (similar to the process to manufacture conventional maltodextrins) to purposefully convert a portion of the normal alpha -1,4 glucose linkages to random 1,2-, 1,3-, and 1,4- alpha or beta linkages. The human digestive system effectively digests only alpha 1,4- linkages; therefore the other linkages render the molecules resistant to digestion. Thus, Fibersol®-2 is GRAS as maltodextrin, resistant to human digestion, and conforms to all working industrial and scientific definitions of dietary fiber.

EXHIBIT B

Matsutani America, Inc.

Fibersol®-2 Nutritional Information

Nutrient	Per 100 grams of ingredient
Total Calories	380 Kcal
Calories from Fat	0 Kcal
Calories from Saturated Fat	0 Kcal
Total Fat	0 g
Saturated Fat	0 g
Polyunsaturated Fat	0 g
Monounsaturated Fat	0 g
Trans Fat	0 g
Cholesterol	0 mg
Sodium	1 mg
Potassium	0 mg
Total Carbohydrate	95.0 g
Dietary Fiber	90.0 g
Soluble Fiber	90.0 g
Insoluble Fiber	0 g
Sugars	5.0 g
Sugar Alcohol	0 g
Other Carbohydrate	0 g
Protein	0 g
Vitamin A	0 IU
Vitamin C	0 mg
Calcium	0 mg
Iron	0 mg

OTHER ESSENTIAL VITAMINS AND MINERALS (per 100 grams)

Thiamine	0 mg	Phosphorus	0.48 mg
Riboflavin	0 mg	Iodine	0 mg
Niacin	0 mg	Magnesium	0.07 mg
Vitamin D	0 IU	Zinc	0 mg
Vitamin E	0 IU	Copper	0.01 mg
Vitamin B-6	0 mg	Biotin	0 mg
Folic Acid	0 mg	Pantothenic Acid	0 mg
Vitamin B-12	0 mg		

This information contained herein is correct to the best of our knowledge. The recommendations or suggestions contained in this bulletin are made without guarantee or representation as to results. We suggest that you evaluate these recommendations and suggestions in your own laboratory prior to use. Our responsibility for claims arising from breach of warranty, negligence or otherwise is limited to the purchase price of the material. Freedom to use any patent owned by Matsutani Chemical Company Ltd. or others is not to be inferred from any statement contained herein.

EXHIBIT C

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1999, p. 5504–5509 Vol. 65, No. 12
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Induction and Transcription Studies of the Dextransucrase Gene in *Leuconostoc mesenteroides* NRRL B-512F

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Received 7 June 1999/Accepted 8 September 1999

Dextransucrase production by *Leuconostoc mesenteroides* NRRL B-512F in media containing carbon sources other than sucrose is reported for the first time. Dextransucrases were analyzed by gel electrophoresis and by an in situ activity assay. Their polymers and acceptor reaction products were also compared by ^{13}C nuclear magnetic resonance and high-performance liquid chromatography techniques, respectively. From these analyses, it was found that, independently of the carbon source, *L. mesenteroides* NRRL B-512F produced dextransucrases of the same size and product specificity. The 5′ ends of dextransucrase mRNAs isolated from cells grown under different culture conditions were identical. Based on this evidence, we conclude that dextransucrases obtained from cells grown on the various carbon sources result from the transcription of the same gene. The control of expression occurs at this level. The low dextransucrase yields from cultures in D-glucose or D-fructose and the enhancement of dextransucrase gene transcription in the presence of sucrose suggest that an activating phenomenon may be involved in the expression mechanism. Dextransucrase mRNA has a size of approximately 4.8 kb, indicating that the gene is located in a monocistronic operon. The transcription start point was localized 34 bp upstream from the ATG start codon. The −10 and −35 sequences found, TATAAT and TTTACA, were highly homologous to the only glycosyltransferase promoter sequence reported for lactic acid bacteria.

Dextransucrases (DS) (EC 2.4.1.5.) are enzymes that transfer the glucosyl moiety from sucrose to acceptor molecules, with a concomitant fructose release. They are used in the synthesis of dextran. In the presence of sucrose and an acceptor like maltose, they synthesize gluco-oligosaccharides (25). Dextran and dextran derivatives have found several valuable applications in the production of fine chemicals such as plasma substitutes and Sephadex. Particularly, gluco-oligosaccharides are used as specialty sugars in the food and cosmetic industries (21).

Several lactic acid bacteria produce DS. Expression is constitutive in *Streptococcus* strains, while it is inducible in *Leuconostoc* strains (8). Until now sucrose has been considered to be the only inducer of DS expression in *Leuconostoc* spp. (32). No gratuitous inducers are known, and the mechanism of DS induction has not yet been reported.

Sugar metabolism in the genus *Leuconostoc* is heterofermentative. When sucrose is used as a carbon source, a specific permease is responsible for its transport into the cell, where it is transformed by sucrose-phosphorylase into fructose and glucose-1-phosphate. The latter is incorporated into the phosphoketolase pathway as glucose-6-phosphate by the action of a mutase, while fructose is excreted to the culture medium (3). Extracellular DS also uses sucrose for dextran production, with additional fructose liberation. When sucrose is depleted, the accumulated fructose is consumed (23, 32).

Leuconostoc mesenteroides NRRL B-512F produces an extracellular DS that synthesizes a soluble polymer, 95% of which is composed of α-(1-6) linkages in the main chain and 5% of which is composed of α-(1-3) branched linkages. Only one DS gene in this strain has been reported (33), while DS has been found in multiple forms of different molecular weights (8, 13, 18). There is insufficient genetic evidence to explain if the various proteins found result from the expression of different genes or from posttranslational modifications. There is no information concerning either the DS gene regulation mechanism or the characterization of the transcript. Although constitutive mutants have been obtained by nonspecific mutation strategies (8, 11, 12), the identification of the promoter region in *L. mesenteroides* DS would allow the construction of constitutive strains by site-directed mutagenesis.

In lactic acid bacteria, some metabolically related genes are organized in clusters or polycistronic operons that are regulated simultaneously (9, 17). Sucrose induces both DS and sucrose-phosphorylase genes in *Leuconostoc*. However, biochemical data support the fact that these enzymes are induced at different stages during fermentation (3). In this work, genetic evidence to elucidate if both genes are under the control of the same promoter is given. In addition, the production of DS from *L. mesenteroides* NRRL B-512F under different induction conditions is examined. Through the isolation and characterization of mRNA, molecular information on the transcript is also provided.

MATERIALS AND METHODS

Strain conditions. *L. mesenteroides* NRRL B-512F was kindly provided by the Northern Regional Research Laboratory (NRRL), Peoria, Ill. Three successive cultures were carried out with each of the various carbon sources (see culture conditions). Cells from the exponential growth phase of the third culture were stored in 15% (wt/vol) glycerol at −20°C and used to inoculate subsequent cultures.

Culture conditions. *L. mesenteroides* was cultured in 100-ml flasks on a rotary shaker at 200 rpm in the standard medium reported by Dols et al. (3) at 25°C

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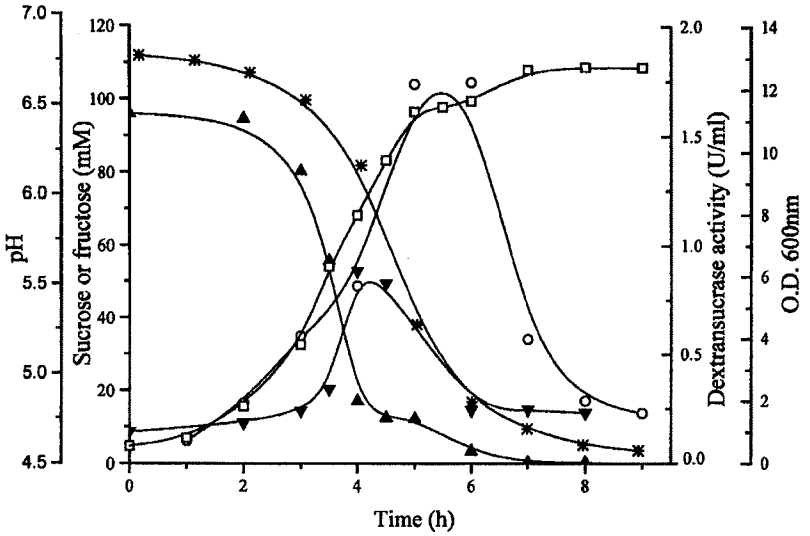

FIG. 1. Batch fermentation profile of *L. mesenteroides* NRRL B-512F under standard conditions at 29°C. ▲, sucrose; ▼, fructose; *, pH; O, DS activity; □, optical density (O.D.).

unless otherwise specified. For cultures grown with other carbon sources, sucrose was replaced by (i) D-glucose, (ii) equimolar quantities of D-fructose and D-glucose, (iii) D-fructose, and (iv) D-xylose (all purchased from Sigma Chemical Co., St. Louis, Mo.). The carbon source concentration was 50 or 117 mM, as specified below. In induction studies, 50 mM fructose cultures were grown until the mid-logarithmic phase was reached. At this point, 1.8 M sucrose was added to obtain a final concentration ranging from 1 to 102 mM.

Biomass measurements. Bacterial growth was estimated by measuring the absorbance at 600 nm. The optical density value was converted to CFU by means of a calibration graph constructed during the culture on each carbon source. CFU were determined after a 24-h cultivation in plate count agar.

DS recovery and assay. After cell removal, the pH was adjusted to 5.2 and the supernatant was filtered through a membrane with a pore size cutoff of 0.2 μm (Millipore Corp., Bedford, Mass.). Subsequently, DS was concentrated by aqueous two-phase partition with 25% (wt/vol) polyethylene glycol 1500 (24). One-half percent dextran T 70 (Sigma) was included in supernatants produced from carbon sources other than sucrose. After centrifugation (7,000 × *g*, 20 min, 4°C), the pellet was dispersed in 20 mM acetate buffer (pH 5.4) and DS activity was measured by monitoring the release of reducing sugars by a 3,5-dinitrosalicylic acid assay (31). One unit of DS activity is defined as the amount of enzyme that produces 1 μmol of fructose per min from a 100-g · liter^{-1} sucrose solution at 30°C in 50 mM sodium acetate buffer (pH 5.4) containing 0.05 g of $CaCl_2$ and 1 g of NaN_3 · liter^{-1}. Specific activity is given as units per gram of total culture protein. Protein was determined after precipitation with 10% (wt/vol) trichloroacetic acid, followed by dispersion in 0.1 N NaOH. Quantification of the soluble proteins was made as described by Lowry et al. (16), with bovine serum albumin as a standard. Unless otherwise specified, all experiments were carried out in triplicate. The variation coefficients were less than 5% in all cases.

Protein electrophoresis and in situ activity analysis. Supernatants from D-glucose and D-fructose cultures were concentrated approximately 40 times by centrifugal ultrafiltration with Centricon 30 tubes (Amicon Inc., Lexington, Mass.). DS from sucrose cultures was analyzed without further concentration. Protein samples were applied in parallel to sodium dodecyl sulfate (SDS)–7% polyacrylamide gels (14). After electrophoresis at a constant current of 30 mA, the gel was cut in two and one-half was stained with Coomassie R-250. The molecular mass was estimated with the High Range SDS-polyacrylamide gel electrophoresis (PAGE) molecular weight standards (Bio-Rad Laboratories, Hercules, Calif.). The other half of the gel was washed and incubated in the presence of sucrose for the in situ DS assay as previously described (20). For a specific levansucrase assay, raffinose was used as a substrate instead of sucrose.

Oligosaccharide and dextran synthesis. Oligosaccharide synthesis was carried out at 30°C with 100 g of sucrose · liter^{-1} and 33.3 g of maltose · liter^{-1} in a solution of 20 mM sodium acetate buffer (pH 5.4) containing 0.05 g of $CaCl_2$ · liter^{-1}, 1 g of NaN_3 · liter^{-1}, and 0.25 U of DS. For dextran synthesis a reaction mixture with the same composition, but lacking maltose, was used. In all cases, DS was inactivated at 75°C.

Carbohydrate analysis. D-Glucose and D-fructose concentrations were determined by an enzymatic UV method (Boehringer Mannheim GmbH, Mannheim, Germany). Sucrose was determined by the same method after treatment with invertase (Sigma). Oligosaccharide analysis was carried out by high-pressure liquid chromatography (HPLC) in a Waters-Millipore C_{18} column equipped with a refractive index detector as previously described (19). Dextran analysis was performed after polymer precipitation with 2 volumes of absolute ethanol; the pellet was recovered by centrifugation and washed three times with deionized water before being freeze-dried. ^{13}C nuclear magnetic resonance (NMR) spectra of the polymer were obtained with an AC300 Bruker spectrometer, at 75.4768 MHz, as described by Dols et al. (4). The chemical shifts were assigned to each carbon according to the method of Seymour et al. (27).

RNA isolation and hybridization analysis. For RNA isolation, 10^9 cells were washed twice and incubated for 30 min at 37°C with 4×10^{-3} mg of lysozyme (Sigma) μl^{-1} and for 1 h with 1% (vol/vol) proteinase K (Boehringer Mannheim GmbH). The isolation procedure was then continued by following the guanidinium thiocyanate method (2) in combination with acidic phenol extraction and treatment with DNase I (amplification grade; Gibco BRL, Rockville, Md.). The molecular weight marker (RNA ladder; Gibco BRL) and 7 μg of total RNA of each sample were separated by electrophoresis with a denaturing formaldehyde-agarose system. Afterwards, the samples were transferred and fixed to a Hybond N nylon membrane (Amersham Corp., Arlington Heights, Ill.) by applying the standard procedure (5). RNA blotted membranes were hybridized according to the manufacturer's instructions with 10 to 20 ng of the DNA probe labeled with ^{32}P by using the Megaprime DNA labeling system (Amersham). The probe was obtained from the *L. mesenteroides* NRRL B-512F DS gene described by Wilke-Douglas et al. (33) (Calgene Inc., Davis, Calif.) after digestion with *SalI* and *NdeI* (Gibco BRL). The enzyme digestion gave one 1.13-kb fragment that includes the region encoding the catalytic domain previously reported (7, 22, 28).

mRNA 5′-end determination. RNA analysis was carried out with the system for rapid amplification of cDNA 5′ ends (Gibco BRL) by following the manufacturer's procedure, which consists of cDNA synthesis and cDNA 3′-end amplification by PCR. cDNA was obtained with Superscript II Reverse Transcriptase (Gibco BRL) and the synthetic oligonucleotide 5′-GATCCGTGAATGCA TACCCG-3′, which is complementary to a conserved sequence in the N-terminal region of the DS gene (33). cDNA 3′ ends were amplified with *Taq* DNA polymerase (Gibco BRL) with the gene-specific primer shown in Fig. 5. The PCR amplification conditions were one cycle of 94°C for 1 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and one final cycle of 72°C for 5 min. The reaction products were purified with a High Pure PCR product purification kit (Boehringer Mannheim GmbH) before being sequenced.

Nucleotide sequence accession numbers. The following accession numbers have been assigned by the EMBL nucleotide sequence database: AJ250903 and AJ250904 (artificial oligonucleotide complementary primer used for gene sequence and oligonucleotide sequence used for the rapid amplification of cDNA 5′ ends, respectively).

RESULTS

***L. mesenteroides* NRRL B-512F DS synthesis with several carbon sources.** Batch fermentation evolution under standard DS production conditions (29°C and 117 mM sucrose) is shown

TABLE 1. DS production in *L. mesenteroides* NRRL B-512F grown with different carbon sources[a]

Carbon source (117 mM)	DS activity (U · mg of protein^{-1})	Production (fold)
D-Xylose	0.005	1
D-Glucose	0.039	8
D-Fructose	0.050	10
D-Glucose + D-fructose	0.104	21
Sucrose	2.390	478

[a] Cells were cultured at 25°C. Activity was measured after the late exponential growth phase. Supernatants were concentrated 40-fold.

TABLE 2. *L. mesenteroides* NRRL B-512F DS production in fructose medium with sucrose addition at the mid-logarithmic stage

Concn (mM) of:		DS activity (U · mg of protein^{-1})
D-Fructose	Sucrose	
50		0
50	1	0.005[a]
50	10	0.013[a]
50	50	0.105[a]
50	102	1.930[a]

[a] Activity measured after 3 h of sucrose addition. All cells were cultured at 25°C.

in Fig. 1. Fructose was released during the first four hours and was later consumed once sucrose was depleted. DS activity reached a maximum of 1.8 U · ml^{-1} at the end of the exponential growth phase, followed by a remarkable decrease in activity closely related to culture acidification.

The effects of different carbon sources on DS production were studied at 25°C to minimize enzyme deactivation. At this temperature, a mean generation time of 1 h was determined for sucrose cultures. DS activity was found in all the concentrated supernatants (Table 1). Final pHs ranged between 5.5 and 6.7 in all cases.

Protein characterization. Electrophoretic analyses were performed with supernatants, with glucose or fructose as carbon sources, and in situ activity assays were carried out to distinguish protein bands able to synthesize a polymer from sucrose. A supernatant obtained under the standard DS production conditions was used as a reference (Fig. 2, lane 1). In the stained gel, two high-molecular-mass bands can be observed (Fig. 2A): an intense band of 170 kDa and a faint one of 160 kDa. Protein profiles after Coomassie staining of proteins from glucose or fructose supernatants were similar to the one from the sucrose culture. After the in situ activity assay was performed (Fig. 2B), two bands of 170 and 116 kDa with polymer-synthesizing activity could be observed (lanes 2, 3, and 4). An additional low-activity band of 160 kDa could be observed in lane 4, and faint bands of 97 kDa were also observed (lanes 2 and 3). While the 170- and 160-kDa bands were distinguished

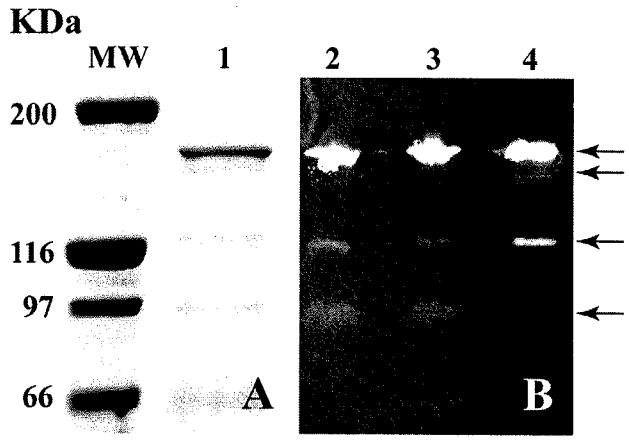

FIG. 2. SDS-PAGE analysis of *L. mesenteroides* NRRL B-512F DS obtained from cells grown with different carbon sources. (A) Coomassie blue-stained gel. Lane MW, molecular mass markers; lane 1, supernatant from sucrose. (B) In situ polymer production from sucrose. Lane 2, supernatants from fructose culture; lane 3, supernatant from glucose culture; lane 4, supernatant from sucrose culture.

after incubation for 24 h, longer incubation times were required to observe the 116- and 97-kDa activity bands.

Analysis of the DS products. The dextran ^{13}C-NMR analysis and the HPLC profile of the acceptor products synthesized by the enzymes obtained from sucrose, fructose, or glucose medium are shown (Fig. 3). It may be observed that the oligosaccharide profile and the polymer structures are the same.

Induction experiments. In order to explore the induction effect of sucrose, *L. mesenteroides* was initially grown under low-level-enzyme-producing conditions with D-fructose, D-glucose, and D-xylose as carbon sources. At the mid-logarithmic stage the cells were washed and transferred to a fresh 117 mM sucrose standard medium for DS production. Appropriate cell densities were reached in order to allow the comparison of results. Before sucrose induction, the highest activity obtained was 0.011 U · mg of protein^{-1}, corresponding to the cells grown in glucose. In all cases, the DS activity was increased after the transfer to the sucrose medium and it could be detected only after 3 h of incubation with sucrose. The highest activity was obtained from cells first grown in xylose (0.569 U · mg of protein^{-1}), while the lowest DS expression was observed when the cells were initially grown in fructose.

Different amounts of sucrose were added directly to fructose cultures, in order to study the sucrose level that is required to induce DS activity (Table 2). It may be observed that large amounts of sucrose (102 mM) are needed to obtain the maximum level of activity. This is 20% less than the level obtained in cultures where the cells were always grown in sucrose (refer also to Table 1).

DS transcription analysis. In order to evaluate the DS messenger in terms of size and level, total RNA was extracted from cells grown under different culture conditions and analyzed by Northern blotting. Well-defined rRNA bands were observed in the denaturing gel (Fig. 4A), indicating a good RNA preparation quality. The hybridization analysis (Fig. 4B) showed that the size of the DS mRNA was approximately 4.8 kb and that the highest concentration was found in the exponential growth phase of the sucrose culture. A fainter hybridization signal was observed in the sample obtained from the lag phase of the same culture. Hybridization bands were not observed for RNA samples from cells grown in alternative sugars and stationary-phase sucrose-grown cultures. They became visible after a longer time exposure, at which time the hybridization signal from the log-phase sucrose culture RNA was extremely high (results not shown).

In order to determine the promoter sequence of the DS gene, the 5′ ends of the mRNA were analyzed by rapid amplification of cDNA 5′ ends. This method is adequate for analyzing traces of mRNA, as with the messenger extracted from cells grown in fructose or xylose. The 5′ ends of the transcripts were compared with the ones obtained from cells

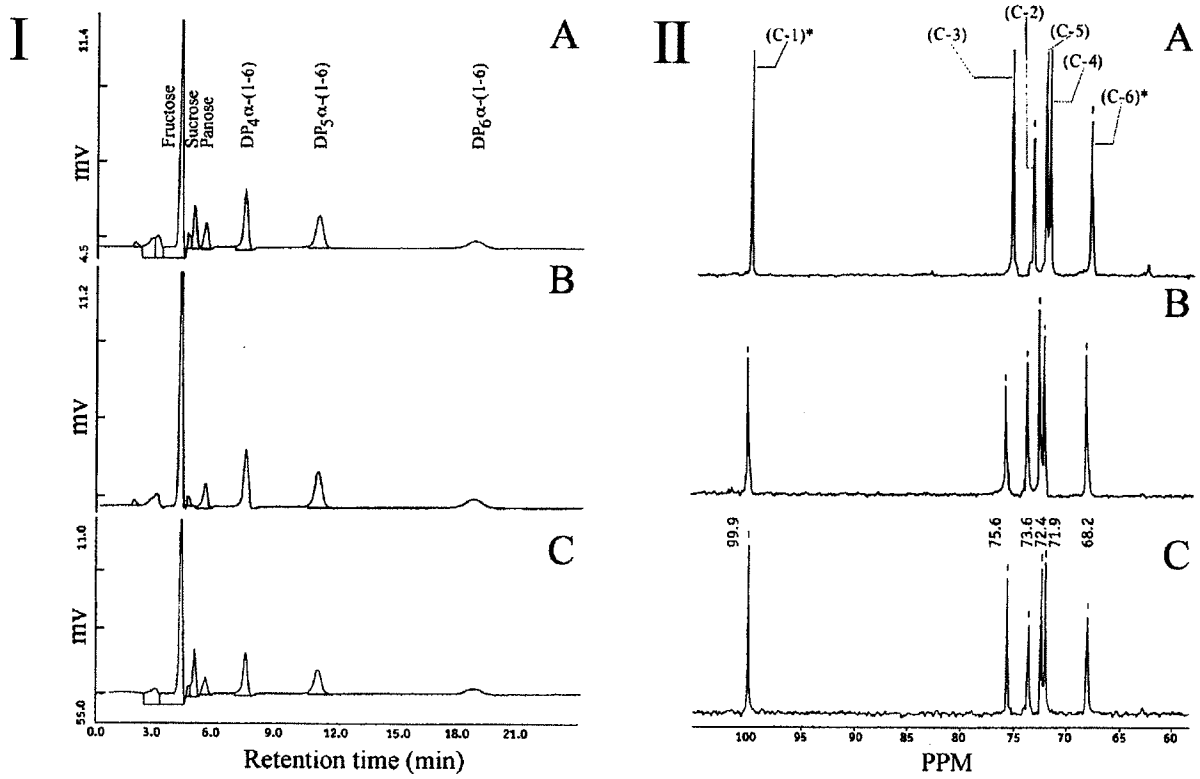

FIG. 3. Analysis of products synthesized by DS obtained from cells grown in fructose (A), glucose (B), and sucrose (C). (Graphs I) HPLC chromatogram of the oligosaccharides produced. These are designated DP_n, with n being the oligosaccharide degree of polymerization (DP). (Graphs II) ^{13}C-NMR spectra of dextran synthesized. *, Carbons involved in the α-(1-6) linkage. Reaction and analysis conditions are reported in Materials and Methods. mv, millivolts.

grown in sucrose. According to the PCR sequencing analysis, it was verified that under the three conditions, the sequences of the 5′ ends of the messengers were the same (Fig. 5).

DISCUSSION

DS yields obtained with *L. mesenteroides* NRRL B-512F grown in sucrose were similar to what has been reported previously (8). An important loss of activity occurred when the pH fell to values that were lower than 5.0 because DS are active in a pH range between 4.8 and 6.2 (18). The results presented here demonstrate that there is a substantial reduction in DS mRNA expression at this moment. Therefore, it may be concluded that at this stage there is activity loss due to enzyme inactivation, which is irreversible according to Miller et al. (18), but also due to the absence of DS gene transcription.

The experiments whose results are reported in Table 1 demonstrate the evident inducing role of sucrose. However, a low-level-induction effect of D-glucose and D-fructose was observed, since DS activities could be detected in the concentrated supernatants. The enzyme yield obtained when D-xylose was used as the carbon source represents the basal DS level. The different enzyme concentration obtained from the cultures with sucrose compared to that of the glucose-fructose mixture might indicate a selectivity difference in the regulatory mechanism. It is interesting that xylose in *Leuconostoc* is assimilated through D-xylulose-5P but that glucose or fructose is assimilated through the phosphoketolase pathway (3). Moreover, as mentioned before, the sucrose uptake pathway differs in its first steps from that of fructose and glucose metabolism. Therefore, differences in enzyme activity might be explained by the presence of a metabolite that plays a role as an activator of gene expression. A molecule involved in the initial sucrose uptake or initial metabolic steps may be such an activator.

It was verified that the main protein bands found in supernatants of all carbon sources studied are DS. The protein of 170 kDa corresponds to the product of the gene described by Wilke-Douglas et al. (33), and the 160-kDa protein corresponds to a DS previously reported (8, 18). We have recently found a very low proteolytic activity in this strain, which could be detected in the concentrated supernatant (26). This result suggests that the 160-kDa band may be produced from digestion of the original 170-kDa protein. The 116- and 97-kDa proteins correspond to levansucrases. This fact was verified with raffinose (specific levansucrase substrate) in an in situ assay, where only these bands were observed (result not shown). Levansucrases of the same molecular mass were also reported by Miller et al. (18). Due to the very small amount of levansucrase, the polymer production was observed only after several days of incubation.

From the analyses of the DS products (dextran and oligosaccharides), it may be concluded that the enzymes obtained in media with different carbon sources have the same specificity. That is, the ^{13}C-NMR spectra of the polymers synthesized with each DS were similar to that of an α-(1→6)-linked linear dextran (20). In all three enzymes, glucosyl is specifically transferred to maltose, producing a series of α-(1-6)-linked oligosaccharides. Accordingly, we conclude that the enzymes obtained in media with different carbon sources are the same in

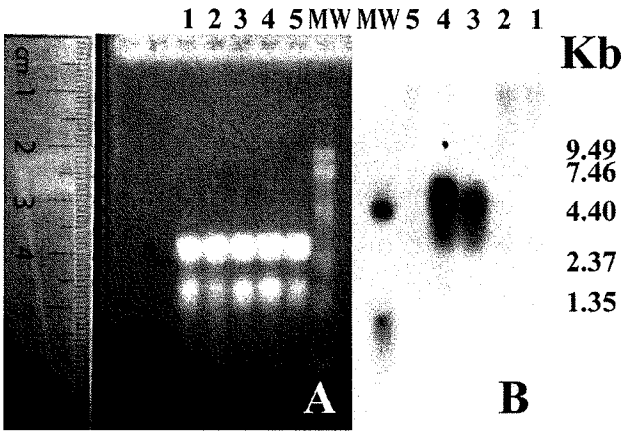

FIG. 4. Analysis of RNA samples extracted from cells grown in fructose (lanes 1), glucose (lanes 2), sucrose lag phase (lanes 3), sucrose log phase (lanes 4), and sucrose stationary phase (lanes 5). (A) Total RNA denaturing formaldehyde-agarose gel electrophoresis. (B) Autoradiogram obtained from the Northern blot. Lanes MW contain the Gibco RNA ladder. In all cases, 7 μg of RNA was analyzed.

terms of protein size and product specificity. It is interesting that although some levansucrase activity was detected in the electrophoretic assay, no levansucrase products were observed in the polymer synthesis reaction, because of the very high dextransucrase/levansucrase ratio.

A classical induction phenomenon requires contact with the cells and the inducer for only a few minutes to allow gene expression. In this case, sucrose behavior as an inducer is atypical since DS activities could be detected only after several hours of contact with sucrose and since the sucrose concentration required to stimulate enzyme production was extremely high (Table 2). These results also show that the growth of three generations in the presence of sucrose was not enough to recover the DS activity levels reached by cells that had always grown in this carbon source.

The correlation between DS mRNA amount and enzyme activity produced under different culture conditions confirms that gene regulation occurs at the transcriptional level. Northern blotting shows that even in the first hour of the sucrose culture, the amount of DS mRNA is considerably higher than the maximum obtained with any other carbon source, a fact supporting the activator hypothesis.

The low enzyme activity of cells transferred to a sucrose medium after growth in fructose may be explained by a fructose repression effect. However, when time-related gene expression was analyzed, it was found that the largest amount of mRNA was observed when 20 mM fructose and 55 mM sucrose were present in the culture, after 3.5 h of fermentation (Fig. 1). These results are consistent with the enzyme production behavior in fed-batch cultures, where in spite of the high fructose concentrations reached, an increase in DS production was obtained. According to López and Monsan, the sucrose concentration should be kept between 15 and 30 mM in order to maintain the microorganism at the maximum growth rate (15). When those results are compared to the ones obtained in this work, it may be concluded that under such culture conditions, the microorganism is also kept at the maximum stage of mRNA synthesis, despite fructose accumulation.

The size of the DS messenger corresponds to the size of the previously reported gene (33), so it is possible to conclude that the DS gene of the B-512F strain is located in a monocistronic operon. This possibility also explains the differences found by Dols et al. (3) in the expression of DS and sucrose-phosphorylase during the culture time, as they claimed that these enzymes were not coinduced by their common substrate. As the 5′ ends of all the analyzed mRNAs were the same, it is concluded that only one gene is transcribed under any culture condition.

Six putative glucosyltransferase promoter sequences have been reported for *Leuconostoc* (19, 33) and *Streptococcus* (1, 6, 10, 29) species. Only one fructosyltransferase promoter sequence, from *Streptococcus mutans*, has been determined experimentally (30). In this work, one transcription start point was found 34 bp upstream from the ATG start codon. The DS promoter presents the sequence TATAAT in the −10 region, which is totally homologous to the conserved region in prokaryotic cells and the reported region for *S. mutans*. The −35 region, TTTACA, presents high homology to the hexamer consensus sequence $T_{82}T_{84}G_{78}A_{65}C_{54}A_{45}$, and the sequence reported for *S. mutans* has four additional base substitutions. The identification of the DS promoter sequence will allow further studies of the gene regulation mechanism in lactic acid bacteria and allow the rational construction of constitutive mutants by site-directed mutagenesis techniques.

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1...  AATAATAATTAACATTGTTAACTTATCACTAGATAAGTTAACAATGTTTTTTTATAAAAAAATTAATGTT

71    ATATAATATTATATAATTCTCTGATTGACTGTTTTTACAATGTTTTTTAAAAATATGTCGATTGTGTATT

141   GAAATTTTTACTGTTTTTACAAATAAATTAAGTTATAATTTTTCAATGTAATAGAAGAGAATATTATAAG
                  -35          -10          mRNA
211   GAGAAAATTTATGCCATTTACAGAAAAAGTAATGCGGAAAAAGCTTTATAAAGTTGGGAAAAGTTGGGTA
      SD       M  P  F  T  E  K  V  M  R  K  K  L  Y  K  V  G  K  S  W  V

281   GTTGGTGGGGTTTGTGCTTTTGCATTAACCGCCTCATTTGCTTTAGCAACACCAAGTGTTTTAGGAGACA
       V  G  G  V  C  A  F  A  L  T  A  S  F  A  L  A  T  P  S  V  L  G  D

351   GTAGTGTACCTGATGTGAGTGCGAATAACGTTCAATCTGCTTCAGATAATACAACGGATACGCAGCAGAA
      S  S  V  P  D  V  S  A  N  N  V  Q  S  A  S  D  N  T  T  D  T  Q  Q  N

421   CACTACGGTTACCGAAGAAAATGATAAAGTACAGTCTGCAGCTACTAATGACAATGTAACAACAGCTGCA
       T  T  V  T  E  E  N  D  K  V  Q  S  A  A  T  N  D  N  V  T  T  A  A

491   AGCGACACAACACAATCTGCTGATAATAATGTGACAGAAAAACAGTCAGATGATCATGCACTTGAT...
       S  D  T  T  Q  S  A  D  N  N  V  T  E  K  Q  S  D  D  H  A  L  D ...
```

FIG. 5. Nucleotide sequence of the N-terminal DS gene and its preceding region. The −10 and −35 promoter regions are underlined, and the transcription start site and direction of transcription are indicated by arrows. SD is the possible ribosome-binding site. The boxed nucleotides correspond to the primer used for PCR amplification of the mRNA 5′ end.

ACKNOWLEDGMENTS

This work was supported by PCP-CONACyT program 39 and by UNAM-PADEP program 5351. M. Quirasco acknowledges the support of an UNAM-DGAPA scholarship.

We also thank M. Vignon for NMR analysis and G. Espin and M. Cevallos for their helpful comments.

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